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Reciprocal regulation of 11-HSDs may predict steroid sensitivity in childhood nephrotic syndrome

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Reciprocal regulation of 11 β -HSDs may predict steroid sensitivity in childhood nephrotic syndrome

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Title of Manuscript:
Reciprocal regulation of 11 β -HSDs may predict steroid sensitivity in childhood nephrotic syndrome

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Short title
11 β -HSDs predict steroid sensitivity in nephrotic synd.

Abbreviations:
GC - Glucocorticoid
GR - Glucocorticoid receptor
NS - Nephrotic syndrome
PBMCs - Peripheral blood mononuclear cells
11 β -HSD - 11beta-hydroxysteroid dehydrogenase

Key words
Steroid resistance, 11 β -HSD

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Contributor's Statement:

Shuji Sai and Masaki Yamamoto: Shuji Sai and Masaki Yamamoto conceptualized and designed the study, drafted the initial manuscript, and approved the final manuscript as submitted. Both authors contributed equally to this study.

Rie Yamaguchi: Dr. Yamaguchi carried out the initial analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Karen E. Chapman: Prof. Chapman helped conceptualize the study, reviewed the manuscript and approved the final manuscript as submitted.

Teruaki Hongo: Dr. Hongo conceptualized and designed the study and approved the final manuscript as submitted.

Review Copy

ABSTRACT

Childhood nephrotic syndrome (NS), in which steroid-dependence occurs concurrently with steroid-resistance, requires aggressive therapy to prevent relapse. Predictive biomarkers that can be used to stratify treatment are urgently needed. Here we report that reciprocal regulation of the glucocorticoid metabolizing enzymes, 11 β -hydroxysteroid dehydrogenase (11 β -HSD) types 1 and 2 is associated with steroid-responsiveness and disease remission in childhood NS, potentially providing a marker to identify patients in which aggressive therapy is required.

Synthetic glucocorticoids (GCs) are used as the first-line treatment for childhood idiopathic nephrotic syndrome (NS). The initial response to steroids is a major prognostic factor in childhood NS.¹ More than 90% of children with minimal change nephrotic syndrome (MCNS) achieve initial remission after an 8-week course of prednisolone on the International Study of Kidney Disease in Children (ISKDC) protocol.² However around 60% of steroid-responsive patients experience frequent relapses with some becoming dependent on steroids to prevent further relapse, but requiring higher doses to do so (exhibiting relative resistance to steroids).² The mechanisms underlying the development of steroid resistance remain unclear and there are currently no reliable biomarkers to identify patients who will go on to develop steroid dependence and resistance. A recent report suggests that low levels of glucocorticoid receptor (GR) in peripheral blood mononuclear cells (PBMCs) are associated with GC resistance in childhood NS.³ However, the density and binding affinity of GRs, as determined by dexamethasone binding assays in PBMCs, were not different in children with steroid sensitive and resistant NS.⁴ Thus, whether GR in PBMCs are a useful biomarker in NS is unclear. We have previously shown that both the type 1 and type 2 isozymes of the pre-receptor GC metabolizing enzyme, 11 β -hydroxysteroid dehydrogenase (11 β -HSD1 and 11 β -HSD2, respectively) are associated with steroid sensitivity in childhood acute lymphoblastic leukemia and indeed, that 11 β -HSD2 may contribute to steroid resistance.^{5,6} Therefore, we hypothesized that 11 β -HSDs in PBMCs could be associated with steroid responsiveness in childhood NS.

CASES AND METHODS

We studied all the Japanese nephrotic syndrome children who were treated at Seirei Hamamatsu General Hospital between April 2013 and October 2015 (a total of 8). Peripheral blood samples were obtained with informed consent. Study protocols were approved by the ethics committee of our hospital. Diagnosis of idiopathic nephrotic syndrome was made by pediatric nephrologists based on clinical and laboratory findings. Patient profiles are shown in Table 1. We had four newly diagnosed and four relapsed NS. Prednisolone treatment was initiated in all patients. For newly diagnosed patients (patients 1, 3, 4 and 8) (Table 1), induction therapy used 2 mg/kg/day prednisolone for 4 weeks (60 mg/m²/day) according to the ISKDC protocol. Because we hypothesized that there would be a qualitative difference between early and late responders, we determined clinical GC-sensitivity/resistance from the initial response to prednisolone treatment at 2 weeks. This is earlier than the common definition (at 4 weeks), but in concordance with a recent report suggesting that the initial response to steroids (at 7 days) is a major prognostic factor in idiopathic NS.¹ Therefore patients 1, 3 and 4, who showed complete resolution of proteinuria (urine protein/creatinine ratio less than 0.2) at 2 weeks and remained negative until 4 weeks, were classified as GC-sensitive. Patient 8 was classified as GC-resistant as she failed to respond to the initial prednisolone treatment. Her histological finding revealed focal segmental glomerulosclerosis on renal biopsy (Table 1).

Of the four relapsing nephrotic syndrome patients, one was classified as infrequent relapse (patient 7). The remaining three (patients 2, 5 and 6) were frequent relapsing nephrotic syndrome patients (FRNS). Modified induction therapy was initiated according to our regional protocol (Nagoya City Kidney Disease Study Group).⁷ According to this protocol, if FRNS patients are not prescribed prednisolone before relapse, to reduce steroid toxicity, 1 mg/kg dose of prednisolone (maximum dose of 30 mg/m²/day) is administered initially, which is lower than the common protocol. Patients already undergoing treatment with ≥ 1

mg/kg prednisolone are started on 2 mg/kg prednisolone daily. For infrequent relapse patient 7, 2 mg/kg prednisolone was administered daily (60 mg/m²/day) until the proteinuria disappeared. We classified patient 7 as GC-resistant due to the presence of proteinuria at 2 weeks, although this had disappeared at 4 weeks. FRNS patients 2, 5 and 6 were all treated with 1 mg/kg prednisolone therapy at relapse.

Blood sampling was carried out before prednisolone treatment and at 2 weeks and 4 weeks after initiating prednisolone treatment. PBMC were isolated by density gradient centrifugation (Ficoll-Paque; Pharmacia). Cells were cultured for 24h in the presence or absence of 10⁻⁶ M dexamethasone in RPMI-1640 with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) (all Invitrogen), at 37 °C, 5% CO₂. RNA was extracted following homogenisation in Trizol (Invitrogen) and resuspended in RNase-free water. RNA (1 µg) was reverse transcribed using SuperScript III (Invitrogen) and quantified by real-time PCR on a LightCycler (Roche) as previously described.^{5,6} All experimental procedures were carried out at Hamamatsu University School of Medicine. Mastermix and primer-probe sets for *HSD11B1*, *HSD11B2*, *NR3C1* (encoding GR) and 18S RNA were purchased from Applied Biosystems. 18S RNA served as the internal control. The fold induction of *HSD11B1*, *HSD11B2* and *NR3C1* mRNA levels were calculated, relative to levels in vehicle treated cells. Values are mean±SEM. Data were analysed in each group using the Wilcoxon matched pairs test. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

HSD11B1, *HSD11B2* and *NR3C1* mRNA were expressed in PBMC in all cases. Absolute levels varied between patients, so findings are expressed as fold induction following dexamethasone. *HSD11B1* mRNA levels were decreased following *in vitro* dexamethasone treatment of PBMC collected from all patients, before or following prednisolone treatment (Fig 1A). The decrease in *HSD11B1* mRNA levels following dexamethasone tended to be

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3 lower in GC-resistant than in GC-sensitive NS, although this did not achieve significance
4 (Fig 1A). In contrast, *HSD11B2* mRNA levels were unchanged by dexamethasone treatment
5 of PBMC collected from GC-sensitive NS patients either prior to or following their
6 prednisolone treatment (the fold induction was not significantly different to 1) (Fig 1B).
7 However, *HSD11B2* mRNA levels were significantly increased by dexamethasone treatment
8 of PBMC collected from GC-resistant NS patients both prior to, and following, their
9 prednisolone therapy at 2 weeks (Fig 1B). The different responses of *HSD11B1* and
10 *HSD11B2* mRNAs following *in vitro* dexamethasone revealed a qualitative difference
11 between GC-sensitive and resistant NS, as seen in GC-sensitive and resistant childhood
12 lymphoblastic leukemia.⁵ The patterns of *HSD11B1* and 2 remained similar during the course
13 of prednisolone therapy, suggesting that GC-sensitivity/resistance is intrinsic in childhood NS.
14 Levels of *NR3C1* mRNA, encoding GR, were unaffected by *in vitro* treatment of PBMC with
15 dexamethasone (Fig 1C).

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32 Expression of 11 β -HSD1 and 11 β -HSD2 is normally reciprocally regulated.⁸ *In vivo*, 11 β -
33 HSD1 predominantly activates endogenous glucocorticoids (converting cortisone to cortisol)
34 whereas 11 β -HSD2 catalyses the reverse reaction, inactivating glucocorticoids. However, it
35 should be noted that 11 β -HSD2 is a high affinity though low capacity enzyme and thus high
36 levels of glucocorticoids can escape inactivation.⁸ The 11 β -HSD enzymes potently modulate
37 exogenous GC action as well. They interconvert prednisolone (active) and prednisone (inert),
38 which itself does not bind to GR.⁸ In many tissues, there is a switch from 11 β -HSD2 to 11 β -
39 HSD1 expression as cells differentiate and mature.^{9,10} The precise mechanism of this
40 reciprocal regulation remains largely unknown, but transcriptional regulator CAAT/enhancer-
41 binding proteins (C/EBPs) are likely involved, as we have previously shown that they
42 regulate 11 β -HSDs in a variety of cell types.^{11,12}

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In normal PBMCs, 11 β -HSD1 expression is low and 11 β -HSD2, negligible.⁸ However in

certain disease conditions, such as early rheumatoid arthritis, levels of mRNA encoding 11 β -HSD2 in PBMC are increased; indeed, 11 β -HSD2 has been reported as a PBMC marker of early rheumatoid arthritis.¹³ Similarly, 11 β -HSD2 is expressed in glucocorticoid-resistant leukemic cell lines where it contributes to prednisolone resistance.⁶ Up-regulation of *HSD11B2* coupled with down-regulation of *HSD11B1* may reflect a return to an earlier developmental state in GC resistant NS. Alternatively, the qualitative difference in 11 β -HSD expression between steroid sensitive and resistant NS may reflect a stable state. This may arise, for example from epigenetic mechanisms¹⁴ which, once established, become self-perpetuating. In steroid-resistant NS, induction of 11 β -HSD2 is predicted to inactivate and thus attenuate prednisolone action, whereas the opposite is predicted for 11 β -HSD1. Thus, in steroid-resistant NS, higher doses of prednisolone are needed to overcome the 11 β -HSD2 inactivation. Therefore we suggest that dexamethasone should be used to treat this condition, rather than prednisolone. The present study has some limitations. We evaluated only Japanese NS patients at our hospital, who may not be representative of childhood idiopathic NS patients in other ethnic groups. Frequent relapse patients were treated with a lower dose of prednisolone (1 mg/kg/day), according to our regional protocol which is intended to reduce steroid toxicity.⁷ Moreover, the sample size is small, and statistical significance was not achieved for the *HSD11B1* mRNA findings. Therefore our findings are preliminary, qualitative rather than quantitative, and require confirmation in a larger number of patients. Nevertheless, they suggest that analysis of expression of these isozymes, and their regulation by glucocorticoids in nephrotic syndrome may aid in tailoring steroid treatment to the patient, by serving as prognostic biomarkers of disease activity.

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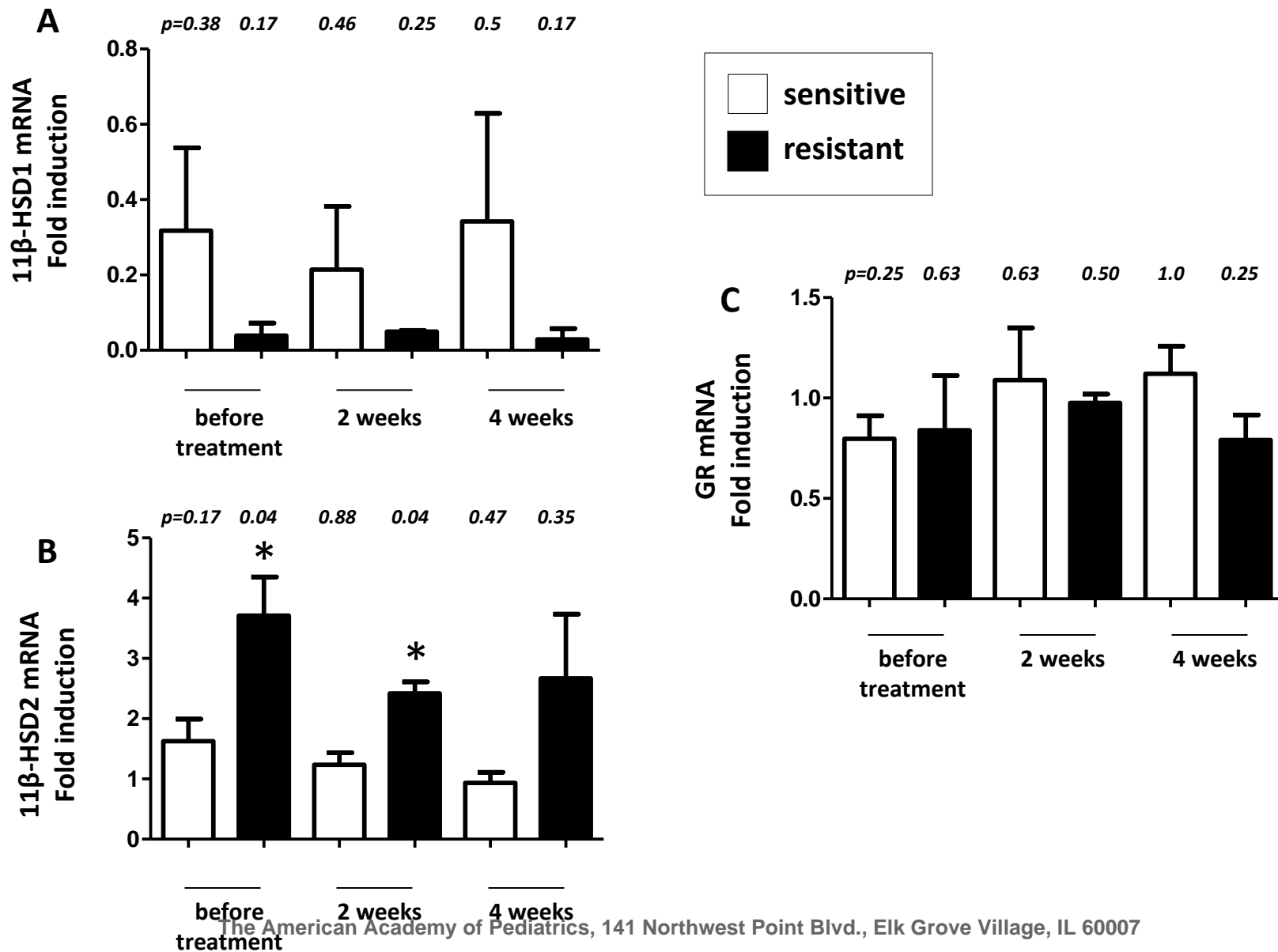
FIGURE LEGENDS

FIGURE 1. Fold induction of 11 β -HSD1/2 and GR mRNA levels in PBMCs in childhood nephrotic syndrome patients during prednisolone treatment. 11 β -HSD1 (A), 11 β -HSD2 (B) and GR mRNA (C) levels relative to before *in vitro* dexamethasone. Open bars indicate GC-sensitive NS and black bars indicate GC-resistant NS. Data are mean \pm SEM. *indicates significant effect of treatment, $p < 0.05$.

Table 1. Patient Profiles

	patient	Age at diagnosis	Disease status	Pathophysiology	Urine protein/creatinine ratio (g/gCr)		
		year/sex	at sampling		before	2 weeks	4 weeks
Glucocorticoid sensitive	1	4/M	diagnosis	n.d.	10.73	0.1	0.08
	2	11/M	relapse	MCNS	6.72	0.04	0.07
	3	2/F	diagnosis	n.d.	13.35	0.05	0.09
	4	6/M	diagnosis	n.d.	3.65	0.03	0.02
Glucocorticoid resistant	5	4/M	relapse	MCNS	2.39	0.07	1.99
	6	13/M	relapse	MCNS	1.94	1	0.03
	7	10/M	relapse	FSGS	17.68	2.89	0.18
	8	3/F	diagnosis	FSGS	6.18	2.03	1.64

Abbreviations: n.d., not determined; MCNS, minimal change nephrotic syndrome; FSGS, focal segmental glomerulosclerosis

Figure 1

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EXAMPLE: Reviewer 1’s comment	EXAMPLE: A brief response to this reviewer’s comment. The text now states: “insert relevant changed text here”	EXAMPLE 1: Page 7, lines 10-22 EXAMPLE 2: No change	
Reviewer 1’s comment 1. The studied cases included 4 steroid resistant and 4 steroid sensitive children. Steroid resistant nephrotic syndrome can not be considered unless the child receive continuous daily dose prednisolone of 2mg/kg for at least 4 weeks without achieving remission. It is not clear how patients were considered steroid responsive or resistant without using the same dose of prednisolone. The authors stated that patients received a dose of 1-2	We apologise that this was unclear. Because we hypothesized that there is a qualitative difference between early and late responders, we determined clinical GC-sensitivity/resistance by the initial response to prednisolone treatment at 2 weeks, which is earlier than the common definition (a recent report showing prognostic value of the early response - at 7d - supports this early definition; Vivarelli et al. 2010 J Pediatr.). Newly diagnosed patients were treated with 2mg/kg prednisolone for 4 weeks, according to the ISKDC protocol. According to our regional protocol, frequent relapsing nephrotic syndrome patients were treated with 1 mg/kg prednisolone at relapse, with the intention of reducing steroid toxicity. The text in Cases and Methods has been amended to make this clear.	Page 4, lines 6 – Page 5, line 5	

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49	mg/kg prednisolone (not a standard dose) and they evaluated their response to steroids after 2 weeks only? Please explain.			
	2. In table 1; patients number 6 and 7 were considered glucocorticoid resistant although their urinary protein to creatinine ratios after 4 weeks of therapy were below 0.2 which is the normal value (0.03 and 0.18 respectively) and thus these 2 patients are steroid sensitive. Please clarify.	As mentioned above, we determined clinical GC-sensitivity/resistance at 2 weeks. We classified GC-sensitive patients as those who showed complete resolution of proteinuria (urine protein/creatinine ratio less than 0.2) at 2 weeks and also remained negative at 4 weeks. If proteinuria was present at 2 weeks, these patients were classified as GC-resistant. This has now been clarified in the Cases and Methods section.	Page 4, lines 19 – Page 5, lines 5	
	3. Used statistical methods and tests should be mentioned.	The data were analysed using the Wilcoxon matched pairs test. This has been added to the Cases and Methods section as well as the Figure Legend.	Page 5, lines 18 - 19	
	4. In Figure 1; HSD11B1, HSD11B2 and NR3C1 expression levels should be illustrated and P values should be written either on the figure if applicable or in the results section.	We did consider this when presenting our data. However, the absolute levels of <i>HSD11B1</i> , <i>HSD11B2</i> and <i>NR3C1</i> mRNA were measured on different days, so variance between patients may reflect differences in assay conditions. However, samples from the same patient were always assayed together, so we have presented the results as the fold induction following dexamethasone treatment. This is now mentioned in the Results and Discussion section. P values have been added to the figure.	Page 5, lines 16 - 23	
	Reviewer 2's comment 1. The authors did not identify inclusion and exclusion criteria for their subjects. Thus it is unclear if these are children with idiopathic nephrotic syndrome in whom they are studying. Further they did not specify the case definition by which they diagnosed these patients with	All childhood NS patients admitted in our hospital for the duration of this study were included, with informed consent. The diagnosis of idiopathic nephrotic syndrome was made by pediatric nephrologists based on clinical and laboratory findings and this information is now included in the Cases and Methods section.	Page 4, lines 1 - 5	

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32	nephrotic syndrome.			
33 34 35 36 37 38 39 40 41 42 43 44	2. Regarding the subjects that they studied, they did not specify the ethnicity of their subjects. Presumably these patients are Japanese, but it is not specified. This is important because the underlying presumption is that the differences in steroid responsiveness is dictated by gene expression levels of HSD11B1 and HSD11B2. If this is the case, then there is a presumption that there is some type of gene variant responsible for the differences in gene expression, epigenetics, or gene-environment interaction. Thus, the underlying gene variants may or may not be present in other populations (which is important in considering the generalizability of the observation to other populations).	Reviewer 2 is correct, all the patients were Japanese. This information has been added to the Cases and Methods section. We also added a comment on this as a study limitation in the Results and Discussion section, as suggested by the Reviewer.	Page 4, lines 1-2 Page 7, lines 13-15	
45 46 47 48 49	3. The medication dosing and clinical response of each case was not described so as to verify the degree of steroid resistance of the non-responders.	Both Reviewers raised this important point. We apologise that this was unclear. For newly diagnosed patients, induction therapy was initiated with 2 mg/kg prednisolone for 4 weeks (60 mg/m ² /day), according to the ISKDC protocol. Relapse patients were treated according to our regional protocol which states that if patients are not prescribed prednisolone before relapse, then to reduce steroid toxicity, initially 1 mg/kg prednisolone (maximum dose of 30 mg/m ² /day) is administered, which is lower than the common protocol. Patients already undergoing treatment with ≥1 mg/kg prednisolone are started on 2 mg/kg prednisolone daily. For the infrequent relapse patient (patient 7), daily	Page 4, lines 6 – Page 5, lines 5	

1 2 3 4 5 6 7 8 9 10 11		prednisolone was administered at a dose of 2 mg/kg (60 mg/m ² /day) until the proteinuria disappeared. The 3 frequently relapsing nephrotic syndrome patients were all treated with 1 mg/kg prednisolone therapy at relapse. We determined clinical GC-sensitivity/resistance by the initial response to prednisolone treatment at 2 weeks, which is earlier than the 4 weeks commonly used as we hypothesized that there is a qualitative difference between early and late responders, and because a recent report has shown the early response (at 7 days) is of prognostic in nephrotic syndrome patients. This information is now included in the Cases and Methods section.		
12 13 14 15 16 17 18 19 20 21 22 23 24	4. In the results, the describe the testing of the mRNA levels for the above genes but do not report the statistics of their results, which one is to presume that they did not detect statistically significant difference in mRNA expression profiles between steroid responders and non-responders.	Data were analysed using the Wilcoxon matched pairs test. This is now mentioned in the Cases and Methods section and also the Figure legend. <i>HSD11B2</i> mRNA levels were significantly increased by dexamethasone treatment of PBMC collected from GC-resistant NS patients both prior to, and following, their prednisolone therapy at 2 weeks. The text of Results and Discussion has been amended to clarify this point.	Page 5, lines 16 – Page 6, lines 7	
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40	5. The write up of the conclusions could be stronger. For example they could have tried to discuss the significance of their findings in the context of the literature they made reference to in their introduction. Furthermore, a discussion of the strengths and weaknesses of the study would have also been appropriate.	We thank the Reviewer for these helpful suggestions. We have incorporated both comments on the significance of our study and discussed its limitations in the revised manuscript.	Page 6, lines 25 – Page 7, lines 22	

Instructions:

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